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Cooperative Effects of Th2 Cytokines and Allergen on Normal and Asthmatic Bronchial Epithelial Cells[1]

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In sensitized individuals, exposure to allergens such as *Dermatophagoides pteronyssinus* (*Der p*) causes Th2 polarization and release of cytokines, including IL-4 and IL-13. Because *Der p* extracts also have direct effects on epithelial cells, we hypothesized that allergen augments the effects of Th2 cytokines by promoting mediator release from the bronchial epithelium in allergic asthma. To test our hypothesis, primary bronchial epithelial cultures were grown from bronchial brushings of normal and atopic asthmatic subjects. RT-PCR showed that each culture expressed IL-4Rα, common γ-chain, and IL-13Rα_1, as well as IL-13Rα_2, which negatively regulates IL-13 signaling; FACS analysis confirmed IL-13Rα_2 protein expression. Exposure of epithelial cultures to either *Der p* extracts, TNF-α, IL-4, or IL-13 enhanced GM-CSF and IL-8 release, and this was partially suppressible by corticosteroids. Simultaneous exposure of the epithelial cultures to IL-4 or IL-13 together with *Der p* resulted in a further increase in cytokine release, which was at least additive. Release of TGF-α was also increased by TNF-α and combinations of IL-4, IL-13, and *Der p*; however, this stimulation was only significant in the asthma-derived cultures. These data suggest that, in an allergic environment, Th2 cytokines and allergen have the potential to sustain airway inflammation through a cooperative effect on cytokine release by the bronchial epithelium. Our novel finding that IL-4, IL-13, and allergen enhance release of TGF-α, a ligand for the epidermal growth factor receptor that stimulates fibroblast proliferation and goblet cell differentiation, provides a potential link between allergen exposure, Th2 cytokines, and airway remodelling in asthma. ***The Journal of Immunology,*** **2002, 169: 407–414.**

Respiratory allergy is due to exposure to aeroallergens, particularly those derived from house dust mites (*Dermatophagoides pteronyssinus* (*Der p*)[4] and *Dermatophagoides farinae*), pets, and fungi. In sensitized individuals, this leads to activation of $CD4^+$ Th memory cells that are responsible for acute and chronic allergic inflammation in asthmatic airways. These cells respond to allergen by production of Th2-type cytokines, including IL-4 and IL-13, which are required to initiate polarization of the Th2 response and drive allergen-specific IgE synthesis by B cells; IL-3, which drives basophil development; and IL-3, IL-5, and GM-CSF, which regulate eosinophil recruitment and survival (1).

In addition to promoting a Th2-type immune response, many inhalant allergens exhibit enzymatic activity, such as the proteolytic activities associated with the major house dust mite Ag *Der p 1*, a cysteine protease, and *Der p 9,* a serine protease (2). In an experimental setting, these proteases disrupt epithelial tight junctions, enhance epithelial apoptosis, and promote the release of a variety of proinflammatory cytokines, including IL-8 and GM-CSF (2, 3). Both disruption of the epithelium and enhancement of cytokine release appear to depend on the proteolytic activity of the allergen because both can be blocked by specific protease inhibitors (3). The functional significance of the direct enzymatic effects of *Der p 1* in the airways has been shown using the highly selective cysteine protein inhibitor PTL11028, which attenuates airway inflammation and bronchial hyperresponsiveness in allergen-sensitized Brown Norway rats (4).

IL-4 and its structural homologue, IL-13, are prominent cytokines in asthma not only on account of their proinflammatory role, but also due to their effects on mucus hypersecretion and airway wall remodelling, as revealed in transgenic animal models (5–9). In vitro studies have confirmed that IL-4 and IL-13 have direct effects on epithelial and fibroblast function. For example, both cytokines enhance mucin expression by bronchial epithelial cells and promote release of GM-CSF and IL-8 from bronchial epithelial or alveolar cell lines (9–11). Using fibroblasts grown from bronchial biopsies of asthmatic subjects, we have recently shown that IL-4 and IL-13 stimulate eotaxin release, but, unlike TGF-β, they are unable to cause transformation of these cells into myofibroblasts and they do not stimulate collagen I gene expression (12). However, because we also demonstrated that IL-4 and IL-13 both enhance release of TGF-β from bronchial epithelial cells, this provides an indirect mechanism through which these cytokines could drive remodelling responses in asthmatic airways.

IL-4 and IL-13 exhibit overlapping, but not identical, effector profiles, which is due to the shared use of the IL-4R α-chain (IL-4Rα) and IL-13Rα_1 in the multimeric IL-4R and IL-13R complex (13–16). IL-4 can also bind to IL-4Rα complexed with the common γ-chain of the IL-2R, while IL-13 can interact with the high affinity IL-13Rα_2, which has a negative influence on IL-13 signal transduction (17, 18). Both IL-4Rα and IL-13Rα_1 are expressed by

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[4] Abbreviations used in this paper: *Der p*, *Dermatophagoides pteronyssinus*; γ_c, common γ-chain.

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bronchial epithelium (18, 19), but expression of IL-13Rα_2 has not been investigated. IL-4 and IL-13 both signal via the transcription factor STAT-6 (14, 20), whose expression is prominent in the bronchial epithelium and is further increased in severe asthma (21). Genetic variation in several components of the IL-4 and IL-13 signal transduction pathway has been implicated in asthma susceptibility or severity (13, 22). For examples, the IL-4Rα gene on chromosome 16p12.1 has eight polymorphisms in the coding region leading to amino acid changes (23, 24). These include an extracellular variant, I50V, that up-regulates receptor responses to IL-4, leading to increased STAT-6 activation and IgE synthesis (25, 26). Q576R and a single nucleotide polymorphism in the intracellular domain located in the STAT-6 binding region enhance signaling and are strongly associated with asthma severity (27).

In view of the ability of allergen to affect epithelial function directly by protease-dependent mechanisms and indirectly through immune cell activation and secretion of Th2 cytokines, the purpose of this study was to analyze the single and combined effects of IL-4, IL-13, and allergen extracts on the secretory profile of normal and asthmatic bronchial epithelial cells. As cell responses may be influenced by the type of receptors expressed, we also analyzed the IL-4R and IL-13R subunits present on bronchial epithelial cells and related their expression to functional outcome.

Materials and Methods

Subjects

For bronchial brushing, nonatopic, nonasthmatic control subjects ($n = 19$) and asthmatic subjects ($n = 19$) were characterized according to symptoms, pulmonary function, and medication. Assessment of asthma severity was in accordance with the Global Initiative for Asthma guidelines on the diagnosis and management of asthma (28). The mild asthmatics were receiving inhaled β_2-agonists (salbutamol) only, while the moderate-severe group was maintained on inhaled corticosteroids, plus or minus long acting β_2-agonists (Table I). All subjects were nonsmokers and were free from respiratory tract infections for a minimum of 4 wk before inclusion to the study. The moderately severe asthmatic subjects treated with inhaled corticosteroids withheld this medication for a minimum of 1 wk before bronchoscopy. Written informed consent was obtained from all volunteers before participation, and ethical approval was obtained from the Joint Ethics Committee of Southampton University Hospital Trust.

All subjects were tested for atopy using a panel of common aeroallergens, and serum IgE levels were measured by standard ELISA. Airway hyperresponsiveness was assessed by histamine inhalation challenge, and expressed as PC_{20} (the cumulative dose of histamine required to produce a fall in forced expiratory volume in 1 s by 20% from baseline).

Fiberoptic bronchoscopy

Bronchial brushings were obtained by bronchoscopy using a fiberoptic bronchoscope (Olympus FB-20D, Tokyo, Japan) in accordance with standard published guidelines (29). Bronchial epithelial cells were obtained using a standard sterile single-sheathed nylon cytology brush (Olympus BC

Table I. *Clinical characteristics of atopic asthmatic subjects and healthy control subjects*

Subject	Age	Sex	Atopy	Asthma Severity	FEV_1[a]	FEV_1[a] % Pred	Histamine PC_{20}
A1	26	M	Yes	Mild	3.78	89	<8
A2	29	F	Yes	Mild	3.07		
A3	19	M	Yes	Mild		91	<8
A4	20	M	Yes	Mild			4
A5	23	M	Yes	Mild	4.2		
A6	21	M	Yes	Mild	2.83	68	0
A7	19	M	Yes	Mild	5.15	100	15.78
A8	37	M	Yes	Mild	3.95	99.7	0.9
A9	27	M	Yes	Mild	4.1	103	0.32
A10	19	F	Yes	Mild	3.05	94	>32
A11	20	F	Yes	Mild	2.9	83	5.7
A12	25	M	Yes	Moderate-severe	3.06	69	.07
A13	21	M	Yes	Moderate-severe	4.9	100	1.8
A14	55	M	Yes	Moderate-severe	2.26	68	0
A15	28	M	Yes	Moderate-severe	3.81	74.3	0.5
A16	39	M	Yes	Moderate-severe	2.06	78	0.76
A17	22	M	Yes	Moderate-severe	4.4	92	0.91
A18	28	F	Yes	Moderate-severe	2.3	73	0.03
A19	21	M	Yes	Moderate-severe	3.6	76	0.62
N1	45	F	No	None	4.7	100	>16
N2	20	M	No	None	5.45	104	>8
N3	21	M	No	None	5.05	100	>8
N4	20	M	No	None	5.55	110	>8
N5	25	M	No	None	3.90	100	>8
N6	33	M	No	None		100	>16
N7	29	F	No	None	3.5	100	>8
N8	21	M	No	None	4.64	100	>8
N9	22	M	No	None	4.7	100	>8
N10	19	M	No	None	5.2	100	>32
N11	21	F	No	None		100	>32
N12	19	F	No	None	3.54	100	>16
N13	20	F	No	None	3.0	78	>16
N14	21	M	No	None	4.8	104	>32
N15	20	M	No	None	5.9	118	>32
N16	34	F	No	None	4.3	107	>8
N17	30	F	No	None	3.6	105	>8
N18	37	F	No	None	3.5	120	>8
N19	25	M	No	None	4.6	106	>8

[a] Forced expiratory volume in 1 s.

9C-26101). This was passed by direct vision via the bronchoscope channel into the lower airways, and five to six consecutive brushings were sampled from the bronchial mucosa of the second and third generation bronchi. Cells were harvested into 5 ml sterile PBS after each brushing. On completion of the procedure, 5 ml RPMI with 10% FBS was added and the sample was centrifuged at 150 × *g* for 5 min to pellet the cell suspension. Epithelial cell purity was assessed by performing differential cell counts on cytospins of the harvested cell suspension.

Epithelial cultures

Primary cultures were established by seeding freshly brushed bronchial epithelial cells into culture dishes containing 3 ml serum-free hormonally supplemented bronchial epithelium growth medium (Clonetics, San Diego, CA) supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin. When confluent, the cells were passaged (p1) using trypsin and were allowed to further expand until used for experimentation at passage 2 or 3; control experiments confirmed that there was no significant difference between the responses of the cells at p2 or p3. Viability was assessed by exclusion of trypan blue dye, and the epithelial nature of cells was assessed by immunohistochemical staining of cultures grown on culture chamber slides (Labtek II eight-well chamber slides; Fisher Scientific, Loughborough, U.K.) using a pan-cytokeratin Ab as well as Abs specific for cytokeratin 13 and 18.

Cytokine release by bronchial epithelial cells

Primary bronchial epithelial cells were seeded into 24-well plates (Nunc; Life Technologies) at a density of 5×10^4/well and allowed to grow to 80–90% confluence. The bronchial epithelium growth medium was then replaced with basal medium (Clonetics) containing insulin, transferrin, and sodium selenite supplement (Sigma-Aldrich, Poole, U.K.), and the cells were rendered quiescent for 24 h before exposure to enzymatically active extracts of house dust mite (*Der p*, 5000 U/ml) (ALK, Copenhagen, Denmark), TNF-α (PeproTech, London, U.K.) (20 ng/ml), IL-4 (20 ng/ml), or IL-13 (20 ng/ml) for 24 h, as detailed in *Results*. Release of IL-8, GM-CSF, or TGF-α into culture supernatants was measured using ELISA kits according to the manufacturer's instructions (IL-8, Pelikine Research Diagnostics, Flanders, NJ; GM-CSF, BioSource, Nivelles, Belgium; TGF-α, Oncogene Research Products, San Diego, CA). Cell number was determined by uptake of methylene blue, and cytokine release was expressed as pg/10^6 cells.

IL-4R and IL-13R analysis

Total RNA was extracted from primary bronchial epithelial cells using TRIzol (Life Technologies, Paisley, U.K.), according to the manufacturer's instructions. Total RNA was extracted from whole blood (1.5 ml) using a RNeasy blood kit (Qiagen, Crawley, U.K.), according to the manufacturer's instructions. RNA integrity was assessed by electrophoresis of the RNA samples on a 1% ethidium bromide-stained agarose gel and observation of intact 28S and 18S ribosomal bands. Reverse transcription was performed for 1 h at 37°C using 1 μg total RNA with 1 μM oligo$(dT)_{15}$ as a primer and 4 U Omniscript Reverse Transcriptase (Qiagen) in the presence of 0.5 mM dNTPs, 10 U RNase inhibitor (Ambion, Austin, TX), and 1× reverse-transcriptase buffer in a total volume of 20 μl. For PCR analysis, 2 μl cDNA was amplified in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCL, 0.2 mM dNTPs, 0.2 μM sense and antisense oligonucleotide primers, and 0.025 U/μl JumpStart *Taq* DNA polymerase (Sigma-Aldrich) in a total volume of 25 μl using a TETRAD thermocycler (MJ Research, Boston, MA). The following $MgCl_2$ concentrations and primers were used: IL-13Rα1, 2 mM $MgCl_2$, sense 5′-TCA TGG TCC CTG GTG TTC-3′ and antisense 5′-CGG TGC GCG ACT CAA CAT AAA-3′; IL-13Rα2, 1.5 mM $MgCl_2$, sense 5′-CAA GGG CAT TGA AGC GAA GA-3′ and antisense 5′-CCA AAT TCC GTC ATC TGA GCA-3′; IL-2Rγ (common γ-chain (γ_c)), 1 mM $MgCl_2$, sense 5′-TAC CGG ACT GAC TGG GAC CAC-3′ and antisense 5′-TGG GGG AAT CTC ACT GAC GA-3′; IL-4Rα, 1.5 mM $MgCl_2$, sense 5′-CTG ACC TGG AGC AAC CCG TAT-3′ and antisense 5′-CCG CTT CTC CCA CTG TGA CCC-3′. After an initial denaturation at 95°C for 1 min and 30 s, samples were amplified using a touchdown protocol for a total of 48 cycles: 94°C, 30 s; X°C, 30 s; and 72°C, 30 s, in which X is 68 for 1 cycle, 67 for 2 cycles, 66 for 3 cycles, 64 for 4 cycles, 62 for 5 cycles, 60 for 6 cycles, and 58 for 28 cycles. Aliquots of PCR products were run on 2% agarose gels and visualized by ethidium bromide staining. All amplicons were designed to span introns and were tested to ensure they would not amplify genomic DNA. The specificity of amplicons was confirmed by direct sequencing using a BigDye dye terminator cycle sequencing (Applied Biosystems, Warrington, U.K.) with the products run on an ABI377 automated sequencer (Applied Biosystems).

Flow cytometry analysis

Epithelial cell cultures were detached from the culture plates using trypsin. The single cell suspension was then washed in PBS containing 2% FCS and resuspended at a concentration of 1×10^7 cells/ml. A total of 100 μl aliquots of this cell suspension was then incubated for 1 h at 4°C with the appropriate primary Abs IL-13Rα2 (Diaclone; IDS, Tyne and Wear, U.K.) and γ_c, and then washed and resuspended in 100 μl PBS/1% BSA containing FITC-conjugated anti-mouse or anti-rabbit secondary Abs. In the case of IL-4Rα, detection was with an IL-4 fluorokine (R&D Systems, Abingdon, U.K.). After 30-min incubation in the dark at 4°C, the cells were washed in cold PBS for analysis using a FACScan flow cytometer (BD Biosciences, Oxford, U.K.) with WinMDI 2.8.

Statistical analyses

Data were analyzed initially using the Kolmogorov-Smirnoff test, which indicated that they were not normally distributed. Therefore, the Wilcoxon Signed Rank test was used for within group comparisons, and the Mann Whitney *U* test was used for between group comparisons. A p value of <0.05 was considered statistically significant. For analysis of cytokine release that involved multiple testing, a Bonferroni correction was applied.

Results

Expression of IL-4R and IL-13R subunits by bronchial epithelial cells

The growth properties of primary cultures of bronchial epithelial cells established from epithelial brushings of normal and asthmatic volunteers are described elsewhere (30). RT-PCR analysis demonstrated that epithelial brushings and cultured epithelial cells expressed mRNA for IL-4Rα, IL-13$R\alpha_1$, IL-13$R\alpha_2$, and the γ_c of the IL-2R (Fig. 1*a*), whereas IL-13$R\alpha_2$ was absent from PBMCs. Cell surface expression of IL-4Rα, IL-13$R\alpha_2$, and γ_c protein was confirmed by FACS analysis using epithelial cultures derived from three normal and three asthmatic subjects. The presence of IL-13$R\alpha_2$ and IL-4Rα was readily detectable in every case; however, in the case of IL-13$R\alpha_2$, levels of expression were found to be variable (Fig. 1*b* and Table II). In contrast, only low levels of γ_c were detectable. IL-13$R\alpha_1$ was not evaluated due to the lack of a suitable Ab for use by FACS analysis.

Effect of Der p *and TNF-α on IL-8 and GM-CSF release by bronchial epithelial cells*

Although TNF-α and the proteolytic activity of *Der p* both have the potential to cause apoptosis (3), neither *Der p* (5000 U/ml) nor TNF-α (10 ng/ml) caused significant induction of apoptosis in the primary cultures at the concentrations used (data not shown). However, exposure of bronchial epithelial cells to *Der p* caused a dose-dependent increase in IL-8 and GM-CSF release by normal and asthmatic bronchial epithelial cell cultures (Fig. 2). For normal cultures, median (range) IL-8 release increased from 2.5 (0.1–8.2) to 7.7 (1.0–59.9) and 11.9 (1.7–59.7) ng/10^6 cells ($n = 12$), and GM-CSF increased from 101 (0–1274) to 128 (0–5358) and 173 (27–5308) pg/10^6 cells in response to 2500 and 5000 U/ml *Der p*, respectively; corresponding values for the asthmatic cultures were 2.9 (0.1–14.5) increasing to 11.9 (0.2–46.9) and 13.9 (0.4–37.7) ng/10^6 cells ($n = 14$) for IL-8, and 200 (0–798) increasing to 375 (42–1708) and 361 (29–1785) pg/10^6 cells for GM-CSF. TNF-α also significantly stimulated IL-8 and GM-CSF release from the epithelial cultures (Fig. 2). In each case, cytokine release was significantly ($p < 0.05$) reduced by the presence of dexamethasone, but, in the case of TNF-α, this remained significantly ($p < 0.05$) above basal levels. There was no significant difference in the magnitude of cytokine release by the normal and asthmatic cultures.

Effect of IL-4, IL-13, and Der p *on IL-8 and GM-CSF release by bronchial epithelial cells*

All experiments were performed using primary epithelial cell cultures, which were >80% confluent and which had been rendered

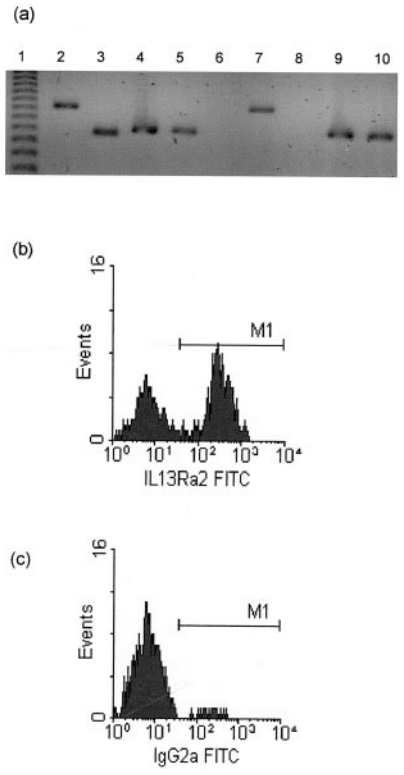

FIGURE 1. *a*, Expression of IL-13Rα_1 (*lanes 2* and *7*), IL-13Rα_2 (*lanes 3* and *8*), IL-4Rα (*lanes 4* and *9*), and IL-2γ_c (*lanes 5* and *10*) in primary bronchial epithelial cells (*lanes 2–5*) and PBMCs (*lanes 7–10*) as detected by RT-PCR. All samples showed a positive signal for G3PDH, which was used as a housekeeping gene (data not shown). *b*, Expression of IL-13Rα_2 on primary bronchial epithelial cells detected using flow cytometry with Abs to IL-13Rα_2 compared with an isotype-matched control Ig (*c*). The bar, M1, indicates the gated cell population from which the data for percentage of fluorescence shown in Table II were derived.

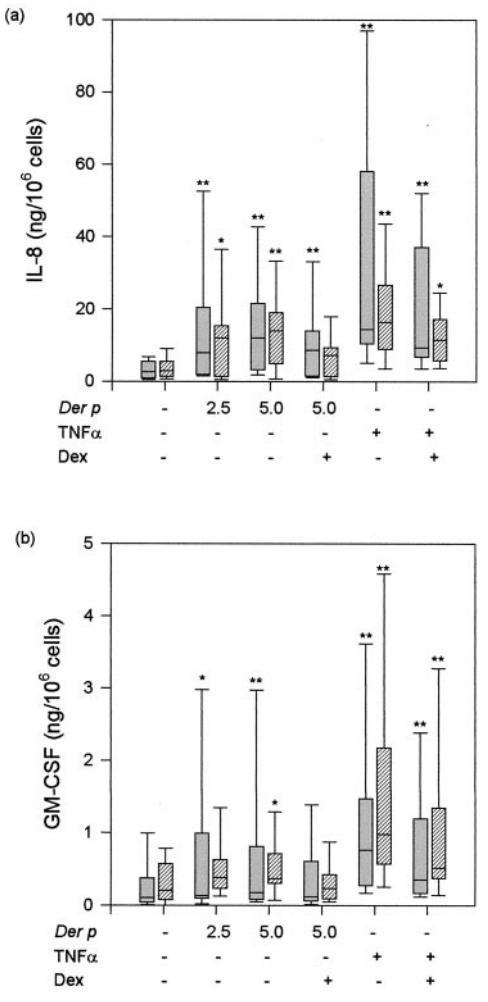

FIGURE 2. IL-8 (*a*) and GM-CSF (*b*) production by primary bronchial epithelial cell cultures of normal (gray bars, $n = 12$) and atopic asthmatic (hatched bars, $n = 14$) subjects after exposure in medium alone or with *Der p* allergen (indicated as 2.5 and 5.0 for 2,500, or 5000 U/ml, respectively) or TNF-α (20 ng/ml) for 24 h in the absence or presence of dexamethasone (10^{-6} M). The box plots show the median and interquartile range, and the bars show the 10th and 90th percentiles. *, $p < 0.05$; **, $p < 0.01$ vs control, untreated cells.

quiescent by growth factor removal. Under these conditions, neither IL-4 nor IL-13 had any significant effect on cell number. In the majority of subjects, exposure of bronchial epithelial cells to IL-4 caused a significant increase in IL-8 and GM-CSF release, and this was suppressible by corticosteroid treatment, irrespective of disease status (Fig. 3). However, in the IL-4-treated asthmatic epithelial cell cultures, the level of IL-8 was still significantly ($p < 0.05$) greater than the untreated control (Fig. 3*a*), even though there was significant ($p < 0.01$) suppression of the IL-4-induced response by dexamethasone. Although IL-13 significantly enhanced IL-8 release, the potency of IL-4 usually exceeded that of IL-13. Similarly, stimulation of GM-CSF release by IL-13 was less than that by IL-4 and failed to reach statistical significance after correcting for multiple testing. When either IL-4 or IL-13 was tested in the presence of *Der p,* release of GM-CSF and IL-8 was enhanced and was at least equivalent to the sum of the amounts released in the presence of either agent alone (Fig. 4).

Table II. *Cell surface of expression of IL-13Rα_2 and IL-4Rα subunits on primary bronchial epithelial cells derived from nonasthmatic (*n = *1–3) and asthmatic (A1–3) subjects*

	% Fluorescence	
Subject	IL-13Rα_2	IL-4Rα
N1	2.1	11.2
N2	9.1	24.2
N3	11.2	16.4
A1	3.6	nd[a]
A2	18.0	33.0
A3	12.7	14.9

[a] nd, Not done.

Effect of Th2 cytokines and Der p *on TGF-α release*

To determine whether IL-4 and IL-13 might affect remodelling responses independently of inflammation, we tested their effects on epithelial release of the potent epithelial and fibroblast mitogen, TGF-α. Using the asthma-derived epithelial cell cultures, significant stimulation of TGF-α occurred in the presence of IL-4, IL-13, or TNF-α, and this was blocked by dexamethasone (Fig. 5*a*). As found for IL-8 and GM-CSF, TGF-α release was also enhanced by *Der p*, and this was further increased in the presence of IL-4 or IL-13 (Fig. 5*b*); however, in this case, the effect was always less than additive. In the 9 of 12 normal epithelial cell cultures, a small increase in TGF-α release was observed under the same treatment conditions; however, this failed to achieve statistical significance even though significant IL-8 release was observed from these cultures (Fig. 5).

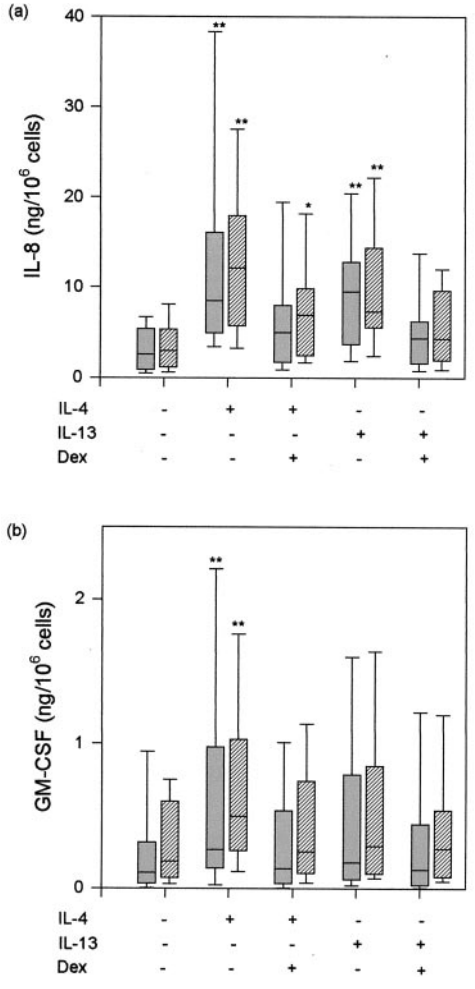

FIGURE 3. IL-8 (*a*) and GM-CSF (*b*) production by primary bronchial epithelial cell cultures of normal (gray bars, $n = 12$) and atopic asthmatic (hatched bars, $n = 14$ (*a*) or 13 (*b*)) subjects after exposure in medium alone or with IL-4 (20 ng/ml) or IL-13 (20 ng/ml) for 24 h in the absence or presence of dexamethasone (10^{-6} M). Data are presented and analyzed as in Fig. 2.

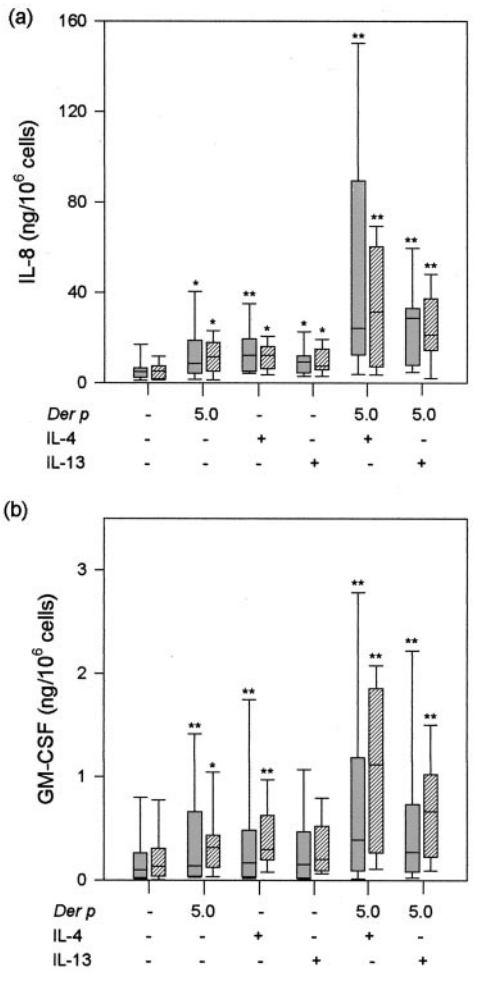

FIGURE 4. IL-8 (*a*) and GM-CSF (*b*) production by primary bronchial epithelial cell cultures of normal (gray bars, $n = 13$) and atopic asthmatic (hatched bars, $n = 15$) subjects after exposure in medium alone or with IL-4 (20 ng/ml) or IL-13 (20 ng/ml) for 24 h in the absence or presence of 5000 U/ml *Der p* allergen. Data are presented and analyzed as in Fig. 2.

Discussion

Bidirectional communication between the resident structural cells and the cells of the innate and adaptive immune system is essential for protection and maintenance of airway function. As the physical barrier to the external environment, the bronchial epithelium occupies a central role in this complex microenvironment by secreting a variety of proinflammatory mediators in response to environmental stimuli and by responding to mediators derived from those immune and inflammatory cells attracted into the airways (31).

Although the effects of IL-4 and IL-13 on epithelial cell lines have been reported previously (9–11), in this study, we provide the first detailed comparative analysis of the responsiveness of normal and asthmatic bronchial epithelial cells. Although experiments with cell lines tend to show reproducible changes in response to stimulation, considerable variation was observed with the primary cell cultures, both at baseline and after stimulation; this most likely reflects differences arising from the outbred human donor population. However, while individual cultures tended to vary in the amount of cytokine released, the overall responses showed consistency in that they tended to move in the same direction, and a donor who had a low response in one assay usually responded weakly in other assays.

In our study, we set out to test the hypothesis that asthmatic bronchial epithelial cells have a heightened response to an allergic environment by comparing responses to IL-4 and IL-13 that would result from the effect of allergen on immune cell function with any direct effects of allergen on epithelial responses. Significantly, even though all atopic asthmatic subjects were sensitive to house dust mite, we found no differences in cytokine release from normal or asthmatic epithelial cell cultures in response to *Der p* and Th2 cytokines, either alone or in combination. This suggests that for cytokine release, there is no difference in epithelial cell sensitivity to the direct effects of inhaled house dust mite allergen in asthma when compared with normal subjects and that, if present, IL-4 or IL-13 will promote IL-8 and GM-CSF release irrespective of disease status. Because all forms of asthma are associated with enhanced production of Th2 cytokines, it seems likely that provision of IL-4 and IL-13 is a key difference between asthmatic and normal subjects. This proposal is consistent with the association between atopy and asthma (32, 33) and with the occurrence of promoter and functional polymorphisms in the genes encoding IL-4 and IL-13 (13), which lead to increased cytokine production or enhanced receptor binding. However, as our experiments were performed at maximally stimulating doses of IL-4, we cannot exclude the possibility that certain single nucleotide polymorphisms in components of the IL-4R signaling cascade that are also associated

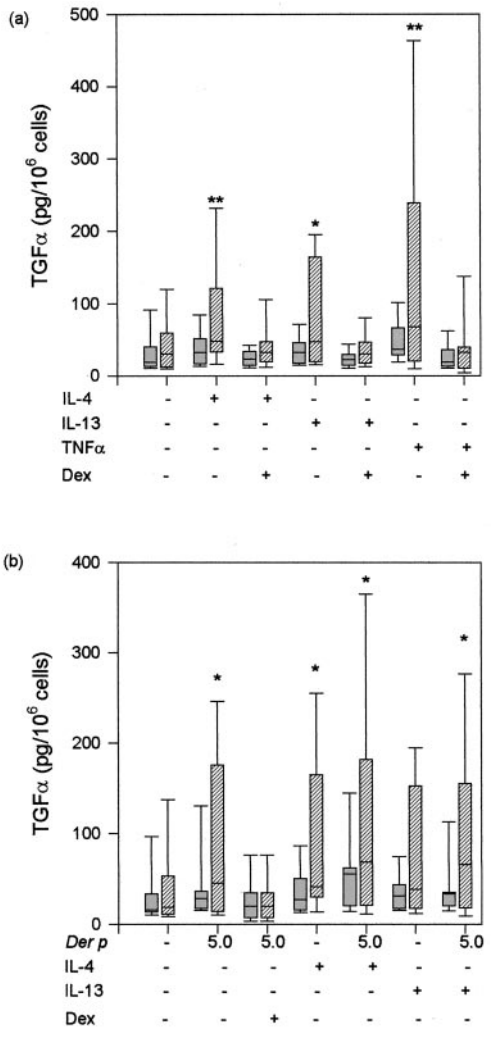

FIGURE 5. *a*, TGF-α production by primary bronchial epithelial cell cultures of normal (gray bars, $n = 12$) and atopic asthmatic (hatched bars, $n = 13$) subjects after exposure in medium alone or with IL-4 (20 ng/ml), IL-13 (20 ng/ml), or TNF-α (20 ng/ml) for 24 h in the absence or presence of dexamethasone (10^{-6} M). In *b*, normal ($n = 11$) or asthmatic bronchial epithelial cells ($n = 11$) were exposed to combination of IL-4, IL-13, and *Der p*. Data are presented and analyzed as in Fig. 2.

with asthma and atopy (13) may also enhance sensitivity to IL-4 when this cytokine is limiting.

The ability of IL-4 and IL-13 to augment cytokine release from bronchial epithelial cells in the presence of *Der p* may provide one explanation for the extent of inflammation in asthmatic airways. Because allergen is universally inhaled, predisposition toward Th2 inflammation in asthmatic airways will amplify epithelial release of GM-CSF and IL-8, leading to exaggerated inflammatory responses in asthma. Consistent with this role, IL-8 is a potent chemoattractant for neutrophils and eosinophils (34, 35) and its levels are increased in asthmatic bronchial epithelium and lavage fluid (36, 37). GM-CSF promotes maturation of dendritic cells and eosinophil survival, and, in an animal model of allergic inflammation, expression of GM-CSF as a transgene in the bronchial epithelium causes persistent inflammation by preventing T cell anergy (38, 39).

In contrast with TNF-α, which gave a consistent increase in cytokine release when used as a control proinflammatory stimulus, the responses of the epithelial cell cultures to IL-4 and IL-13 were much more variable, with some cultures failing to show any response to these cytokines. Although several polymorphisms have been identified, which have positive effects on IL-4 and IL-13 signaling, this system is also tightly controlled by a variety of negative regulators. These include SHP phosphatase, silencing of cytokine signaling proteins (40–43), a dominant-negative STAT-6 splice variant whose expression we have recently described in normal and asthmatic bronchial epithelial cells (21), and IL-13Rα_2 (18). The latter appears to negatively regulate IL-13 function by competing for IL-13 binding and promoting receptor internalization (17). In our study, we provide the first evidence that this receptor is expressed in human bronchial epithelium, suggesting that it may be involved in regulating epithelial responses to IL-13 and IL-4. Although we did not undertake a systematic analysis of surface IL-13Rα_2 expression in each of the cultures that we studied, it is interesting that of the six individuals analyzed, the culture that expressed the highest level of IL-13Rα_2 failed to respond to IL-13 and IL-4 in the face of a normal response to TNF-α (fold stimulation above baseline using IL-4, IL-13, and TNF-α as stimuli was 0.6, 0.2, and 6.0, respectively, for IL-8, and 1.1, 0.6, and 2.2 for GM-CSF). This suggests that IL-13Rα_2 may be an important modifier of epithelial responses to Th2 cytokines and that further work examining the function and regulation of IL-13Rα_2 expression in normal and asthmatic bronchial epithelium is warranted.

The lack of any overall significant difference between responses of normal and asthmatic bronchial epithelial cells to *Der p* and IL-4 or IL-13 differs from a previous study that reported that asthmatic bronchial epithelial cells are more sensitive to diesel exhaust particles when assessed by release of GM-CSF, IL-8, and RANTES (44, 45). This suggests that some epithelial responses are regulated by conditions in the airway microenvironment (e.g., the provision of Th2 cytokines), while other responses are fundamental to the nature of the asthmatic epithelium. Because we have found that asthmatic bronchial epithelial cells are more sensitive to oxidant-induced apoptosis (30), it seems likely that agents such as diesel exhaust particles, which have the capacity to generate reactive oxygen, impact on an underlying difference in the ability of asthmatic epithelial cells to deal with oxidant stress provided by environmental pollutants. However, it is also evident that Th2 inflammation has the potential to impinge on this susceptibility through stimulation of endogenous, inflammatory cell-derived oxidants.

In addition to their proinflammatory role, IL-4 and IL-13 have been implicated in goblet cell metaplasia by enhancing IL-8 release that causes oxidant-induced activation of the epidermal growth factor receptor as a consequence of attraction of neutrophils into the epithelium (46). However, as IL-4 has been shown to affect (mucin 5 subtypes A and C) expression in the absence of neutrophils in vitro (9), we explored the possibility that IL-4 and IL-13 may activate the epidermal growth factor receptor directly by increasing release of TGF-α, one of its activating ligands. Recent studies have implicated TGF-α in the process of retinoic acid-induced goblet cell differentiation in air-liquid interface cultures grown in the presence of IL-13 (47). TGF-α release was readily detected in asthmatic cell culture supernatants, while for most of the normal cell supernatants, TGF-α levels were close to the limit of detection. Consistent with its effects in hepatocytes (48), we found that TNF-α also increased TGF-α release into asthmatic epithelial cell culture supernatants. Because neutrophils, mast cells, and macrophages are all sources of TNF-α, this finding suggests a novel mechanism whereby TNF-α can make a significant contribution to remodelling responses via TGF-α mobilization.

In contrast with IL-8 and GM-CSF, which were released with similar efficacies from normal and asthmatic epithelial cells, no

significant stimulation of TGF-α release was detected in the nonasthmatic culture supernatants under any of the conditions tested; however, there was a trend for increased release. Failure to achieve statistical significance probably reflects the lower levels of TGF-α detectable in the normal epithelial cell culture supernatants and may be due to a difference in the expression, processing, or use of TGF-α, rather than to any selective suppression of responses to the Th2 cytokines. Consistent with this proposal, IL-4 and IL-13 were found to stimulate IL-8 release even though the same cultures failed to respond with TGF-α release. TGF-α is synthesized as a transmembrane precursor whose cleavage is catalyzed by metalloproteases including TNF-α-converting enzyme (49). Thus, failure to detect TGF-α in nonasthmatic culture supernatants may be due to either low TGF-α gene expression or failure to process and release the growth factor (or a combination of both factors). Alternatively, as epithelial cells express epidermal growth factor receptors, TGF-α may be synthesized and cleaved equivalently by normal and asthmatic cell cultures, but it may be used more rapidly by the nonasthmatic cell cultures, resulting in an apparent lack of growth factor release. Systematic analysis of each of these processes will be required to determine the underlying cause of this potentially important difference between the normal and asthmatic epithelial cells. On the one hand, excessive production of TGF-α in asthma may contribute to airway remodelling by affecting both goblet cell metaplasia and fibroblast proliferation, whereas in contrast, failure of asthmatic epithelial cells to use TGF-α may underlie an abnormal repair response in asthma and explain the extent of epithelial disruption that is characteristic of this disease.

Our finding that *Der p* enhances release of TGF-α from epithelial cells has never been reported previously. *Der p* allergens are known to contain cysteine and serine protease activity (50); however, it is not known whether they directly cleave the TGF-α precursor or activate cell surface metalloproteases. As many growth factors are produced as cell surface precursors, it will be important to determine to what extent allergen-derived proteases modify the cell surface leading to production of biologically active molecules, as well as causing disruption of adhesion junction proteins.

In conclusion, we have provided evidence for functional interactions between allergen and Th2 cytokines in the bronchial epithelium in asthma linked to proinflammatory and remodelling responses. Our data identify the IL-13Rα_2 as a potential regulator of IL-13 and IL-4 signaling in these cells and suggest an important phenotypic difference between normal and asthmatic epithelial cells linked to TGF-α metabolism. The availability of a reliable method for the growth of primary cultures from normal and asthmatic bronchial epithelium should now pave the way toward dissection of the underlying mechanisms that control bronchial epithelial cell function in an allergic environment.

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